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# Microplate hybridization assay for detection of isoniazid resistance in *Mycobacterium tuberculosis*

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**Early and accurate detection of drug resistant *Mycobacterium tuberculosis* can improve both the treatment outcome and public health control of tuberculosis. A number of molecular-based techniques have been developed including ones using probe molecules that target drug resistance-related mutations. Although these techniques are highly specific and sensitive, mixed signals can be obtained when the drug resistant isolates are mixed with drug susceptible isolates. In order to overcome this problem, we developed a new drug susceptibility test (DST) for one of the most effective anti-tuberculosis drug, isoniazid. This technique employed a microplate hybridization assay that quantified signals from each probe molecule, and was evaluated using clinical isolates. The evaluation analysis clearly showed that the microplate hybridization assay was an accurate and rapid method that overcame the limitations of DST based on conventional molecular techniques. [BMB reports 2009; 42(2): 81-85]**

## INTRODUCTION

WHO (World Health Organization) has estimated that up to one third of the world's population is infected with *Mycobacterium tuberculosis*, and that 8.8 million people worldwide are newly infected each year resulting in approximately 1.6 million deaths annually (1). The global spread of tuberculosis (TB) is further complicated by the increasing appearance of multidrug-resistant (MDR) *M. tuberculosis*. MDR strains are defined as being resistant to isoniazid (INH) and rifampin (RIF), which presently comprise the backbone of anti-TB chemotherapy. Their spread of MDR strains is a serious threat to tuberculosis control programs through out the world.

Early and accurate detection of resistant strains allows for the initiation of modified treatment regimens, which would improve both treatment outcome of patients and public health control by minimizing the transmission of drug-resistant strains. The determination of drug resistance using phenotypic method, *i.e.*, conventional culture-based drug susceptibility tests (DST), provides definitive results but routinely takes 6 to 10 weeks (2). In order to shorten this period, considerable interest has been generated toward developing molecular based DSTs particularly targeting RIF and INH (3-5).

Although these molecular techniques seem to be rapid and highly specific, the sensitivity of the techniques involved is not always to be 100% accurate compared to conventional DST using culture methods. The reason for the problem seems to arise from the difficulty of molecular assays to differentiate mixed signals from infections with mixed strains. In the case of mixed strains, background signals are obtained from experiments that are often carried out with inappropriate stringency. In addition to this, the detection limits of molecular techniques are lower than that of conventional culture-based DST. According to the Löwenstein-Jensen (LJ)-based proportional method, the strains are considered to be resistant to anti-tuberculosis drug when the ratio of the number of the bacteria colonies on media containing antibiotics to that of control on media without antibiotics is equal to or greater than 1% at the conventional culture-based method (6). For these reasons, it is critical to develop a molecular method that can determine the mixed population of the bacteria quantitatively and qualitatively.

In an attempt to overcome these limitations, we developed a microplate hybridization assay, which can generate signals in a quantitative and qualitative manner to develop DST to INH.

## RESULTS

### Development of microplate hybridization assay for DST of *M. tuberculosis* to INH

The microplate hybridization assay developed in this study was designed to detect point mutations at the *katG* codon 315 and the upstream promoter sequence of *inhA* at -15 positions, which are highly related to INH resistance of *M. tuberculosis*.

Fig. 1 shows the representative results of the assay using strains of *M. tuberculosis* whose genotypes have been analyzed. In short, the results showed that microplate hybridization assay using probes for detecting single point mutations at each site were successful. That is, *katG* 315 wild type probe

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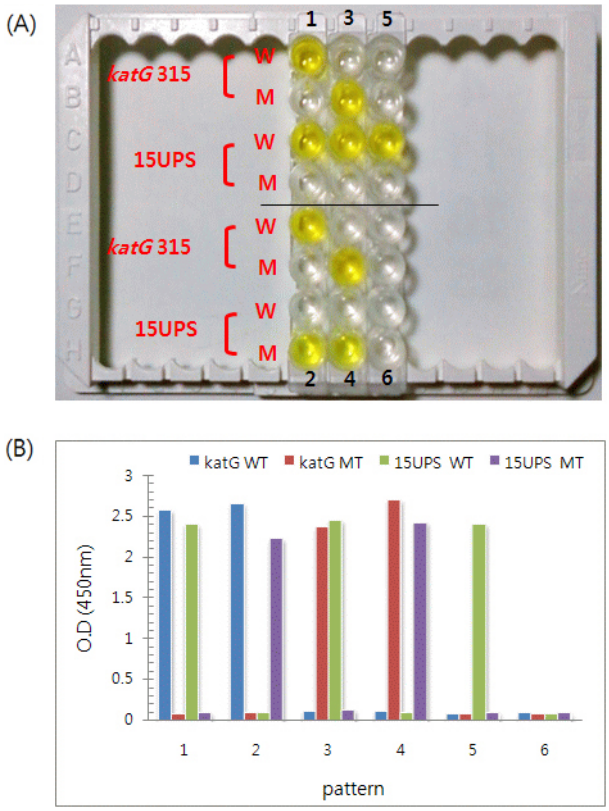


**Fig. 1.** Representative patterns of clinical isolates obtained by microplate hybridization assay. (A) Representative results obtained from hybridization of the microplate. The positions of the oligonucleotides are given on the left side. (B) The results were assessed by reading absorbance values at 450 nm. Pattern 1, strain with the *katG* $315^{WT}$/15 $UPS^{WT}$; pattern 2, strain with the *katG* $315^{WT}$/15 $UPS^{MT}$; pattern 3, strain with the *katG* $315^{MT}$/15 $UPS^{WT}$; pattern 4, strain with the *katG* $315^{MT}$/15 $UPS^{MT}$; pattern 5, strain with the *katG* 315 gene deletion or strain carried the other mutations in *katG* 315 and 15 UPS mutant type; pattern 6 is the negative control. W: wild type, M: mutant type, NC: negative control. The experiments were performed at least three times, and the result of one representative experiment is shown.

(*katG* 315 WT) hybridized to a strain that contained wild type sequences at the *katG* 315 site, whereas the same probe did not hybridize to a reference strain that contained mutated sequences at the *katG* 315 site. In addition, the *katG* 315 mutant probe (*katG* 315 MT), which was designed to detect the *katG* 315 AGC → ACC mutation, hybridized to a reference strain that contained AGC → ACC mutation at the *katG* 315 but not to a reference strain that contained wild type sequences at the *katG* 315 site. Likewise, the wild type probe to -15 site of upstream promoter sequences (15 UPS) of *inhA* hybridized to a reference strain that contained wild type sequences at the 15 UPS wild type sequences, whereas it did not hybridize to a mutated sequences of the 15 UPS. On the contrary, the probe designed to detect C → T change at 15 UPS hybridized to a reference strain that contained C → T change at 15 UPS site, and not to a reference strain that contained wild type sequences at the 15 UPS site.

### Comparison of DST result using microplate hybridization assay with conventional culture-based DST

In order to evaluate the sensitivity and the specificity of DST using the microplate hybridization method, 92 INH-resistant and 29 INH-susceptible *M. tuberculosis* isolates that were determined by conventional culture-based DST were analyzed. The mutations at *katG* 315 were detected in 47 cases among 92 INH-resistant isolates, which were determined by not hybridizing to the *katG* 315 WT probes (Table 1). Among these, 45 isolates hybridized to *katG* 315 MT indicating the presence of *katG* 315 AGC → ACC mutations in INH-resistant clinical isolates. Subsequent sequence analysis revealed that all the 45 isolates studied possessed a *katG* 315 AGC → ACC substitution. The remaining two isolates did not hybridize to *katG* 315 WT probes and the *katG* 315 MT probes. Sequence analysis confirmed that one had the AGC → AAC mutation while the other had a total deletion of the *katG* gene. On the other hand, all the 29 INH-susceptible *M. tuberculosis* isolates hybridized to the *katG* 315 WT probes, indicating no sequence alterations at that site in these strains.

**Table 1.** Microplate hybridization assay results for the detection of mutations conferring INH resistance in comparison with DNA sequencing data of INH-resistant and INH-susceptible strains

| Target gene | Phenotypic pattern | Microplate hybridization assay | DNA sequencing | No. of isolates |
|---|---|---|---|---|
| *katG* 315 | R[a] | WT (+), MT (−) | No mutation | 45 |
| | | WT (−), MT (+) | AGC → ACC (Ser → Thr) | 45 |
| | | WT (−), MT (−) | AGC → AAC (Ser → Asn) | 1 |
| | | | Total deletion of *katG* gene | 1 |
| | S[b] | WT (+), MT (−) | No mutation | 29 |
| 15 UPS | R | WT (+), MT (−) | No mutation | 68 |
| | | WT (−), MT (+) | −15 C → T | 22 |
| | | WT (+), MT (+) | −15 C → T + No mutation | 2 |
| | S | WT (+), MT (−) | No mutation | 29 |

Cut off value = means ± SD. *a*: INH-resistance, *b*: INH-susceptible

The mutations at 15 UPS were detected in 24 cases among 92 INH-resistant isolates by using the 15 UPS mutant probe (15UPS MT) (Table 1). All the isolates that hybridized to 15UPS MT were confirmed to carry a base substitution C to T at 15 UPS site by DNA sequence analysis. On the other hand, 2 of 24 hybridized both to 15UPS MT and 15UPS WT probe at the same time. The sequence analysis of both these isolates showed that two genotypically different populations of *M. tuberculosis* isolates existed having wild type and mutant type C → T sequences indicating the presence of both INH-resistant stains and INH-susceptible strains.

Then, the detection rate of the microplate hybridization assay, when genomic DNA containing drug resistance related sequence alterations were mixed with wild type genomic DNA was evaluated (Fig. 2). For this, genomic DNA with mutated sequences was mixed with wild type genomic DNA in ratios of 1:99 to 10:90, and then the microplate hybridization assay was performed using the mutant probes. The results showed that the microplate hybridization assay was able to detect genomic DNA with mutated sequences even at a ratio of 1:99. This indicated that the microplate hybridization assay could successfully detect 1% population of drug resistant clinical isolates mixed with 99% drug-susceptible isolates. This implicated the microplate hybridization assay to be as sensitive as culture-based DST which detects the drug resistance of *M. tuberculosis* when the proportion of resistant clinical isolates are more than 1%.

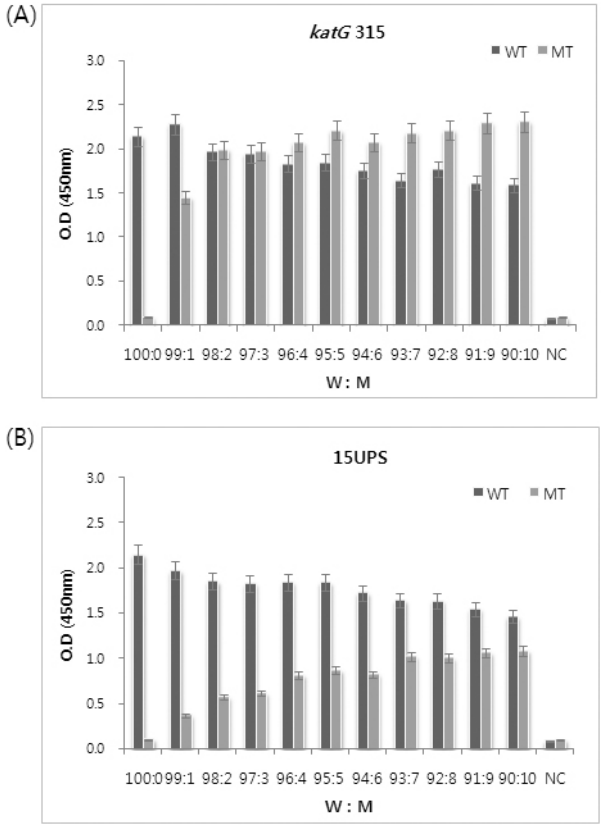


**Fig. 2.** Detection rates of mutant type genomic DNA that mixed with wild type genomic DNA. The mutant type genomic DNAs were used at ratios ranging from 1-10%. (A) Genomic DNA that was mixed with wild and mutant type of *katG* 315 genes was used. (B) Genomic DNA that was mixed with wild and mutant type of 15 UPS was used. Microplate hybridization assay detected the *katG* 315 and the 15 UPS mutant type genomic DNA from the ratio of 1% mixed with wild type gene respectively. The total template concentration used was 2 ng. W[a]: wild type genomic DNA, M[b]: mutant type genomic DNA.

**Sensitivity and specificity of the microplate hybridization assay**

To determine the sensitivity of each probe, 1 ng to 10 fg of wild type and mutant type DNA of *katG* 315 and 15 UPS were used. In the microplate hybridization assay, positive signals were obtained with 1 pg to 10pg at the *katG* 315 wild type, 15 UPS wild type and mutant type probe, and it was obtained with 100 fg to 1 pg at the *katG* 315 mutant type probe (data not shown).

By using the microplate hybridization assay, a total of the INH-resistant clinical isolates 68 (74%) INH-resistant *M. tuberculosis* was confirmed as INH resistance and all the 29 (100%) INH-susceptible *M. tuberculosis* was confirmed as INH susceptible. These results were confirmed by sequence analysis to determine the accuracy of the assay. The results revealed that all the mutant type and the wild type hybridization patterns of the *katG* 315 and the 15 UPS were correctly concordant with the sequencing data (Table 2). Therefore, the overall sensitivity and specificity of the microplate hybridization assay were 74% based on the conventional method and 100% based on the sequence analysis, respectively.

## DISCUSSION

In the management of mycobacterial infections, the rapid detection and appropriate treatment with susceptible drugs are

**Table 2.** Comparison of microplate hybridization assay results and DNA sequencing data for the detection of *M. tuberculosis* with mutation in *katG* 315 and 15 UPS

| Isolates (No.) | | | Microplate hybridization assay results | | | |
|---|---|---|---|---|---|---|
| | | | *katG* 315 | | 15 UPS | |
| | | | WT (%) | MT (%) | WT (%) | MT (%) |
| Sequencing results (92) | *katG* 315 | WT[a] (45) | 45 (100) | 0 (100) | - | - |
| | | MT[b] (47) | 0 (100) | 47 (100) | - | - |
| | 15 UPS | WT (68) | - | - | 68 (100) | 0 (100) |
| | | MT (24) | - | - | 0 (100) | 24 (100) |

*a*: wild type, *b*: mutant type

crucial. These goals can be achieved by improving the diagnosis of drug-resistant tuberculosis using molecular methods. In the present study, a microplate hybridization assay for early and accurate detection of INH resistance in *M. tuberculosis* was established. The probes were able to screen wild and mutant genotypes at *katG* 315 and 15 UPS. The assay was performed on 92 INH-resistant and 29 INH-susceptible isolates. By use of the microplate hybridization assay, the mutations at *katG* 315 were accurately detected in 51% (47/92) INH-resistant *M. tuberculosis* isolates, similar to that previously reported. Among them, 45 isolates had Ser315Thr amino acid replacement and one of the remaining two isolates had an AGC → AAC (Ser → Asn) mutation, while another had a total deletion of the *katG* gene, respectively. Mutation in the *katG* 315 (AGC → AAC) and total deletion of the *katG* gene seem to be rare, but have previously been reported (7).

The base substitution (C → T) at 15 UPS was detected in 26% (24/92) INH-resistant isolates. Among these, two isolates were confirmed to possess INH resistance mixed with susceptible strains. In this case, the microplate hybridization assay detected both strains simultaneously. Furthermore, it was able to detect less than 1% of mutant type DNA in the mixture. These results have important implications compared to other molecular approaches, which have a detection limit of 10% mutant type DNA in a mixture of wild type and mutant type DNA.

By analyzing the *katG* 315 gene and 15 UPS, 74% of the INH-resistant *M. tuberculosis* were detected. This detection rate is very high, as approximately 10 to 25% of INH-resistant strains do not contain mutations in any known gene targets for INH resistance (8, 9). In addition, 100% of the INH-susceptible *M. tuberculosis* was confirmed as INH susceptible strains. When the mutant type and the wild type hybridization patterns of the microplate hybridization assay were compared to DNA sequencing, all the results were correctly concordant with the sequencing data.

The microplate hybridization assay has several advantages compared to conventional oligonucleotide hybridization assays. Firstly, the assay is easy to perform and interpret, and it utilizes multipurpose equipments that are available in any basic molecular biology laboratory and could therefore be performed in routine clinical microbiology laboratories. Secondly, with this assay, INH resistance in *M. tuberculosis* isolates can be accurately predicted within just 4 hours after PCR amplification, and can therefore shorten the regular laboratory turnaround time for INH susceptibility testing. Thirdly, the assay made it possible to handle a greater number of samples at the same time, thus leading to the development of an automated assay procedure for detection of INH resistance. Most importantly, when the clinical isolates were mixed with drug-resistant and susceptible strains, the microplate hybridization assay successfully detected both strains simultaneously up to the level of conventional culture-based DST methods. Therefore, the microplate hybridization assay has proven to be accurate, rapid and simple, and also overcomes the limitations of conventional-molecular based DST.

On the other hand, one must be aware that the microplate hybridization assay has the limitations of other molecular tests for the detection of antibiotic resistance, and therefore, it may not be able to totally replace the traditional culture-based method for DST. This is basically due to the fact that none of the molecular tests previously established has targeted all possible genes or mechanisms involved in resistance, and thus, a variable proportion of resistant stains will not be detected. The second inherent limitation of this newly developed technique is that it utilizes DNA extraction, which is isolated from cultured bacteria. Thus, information about the molecular mechanisms of INH resistance and study for direct application to clinical specimens were required to be used routinely in clinical laboratories.

## MATERIALS AND METHODS

### Clinical isolates of *M. tuberculosis*

One hundred and twenty one clinical isolates of *M. tuberculosis* consisting of 92 INH-resistant and 29 INH-susceptible isolates were used in this study. All the clinical isolates and clinical drug resistance-susceptibility profiles of these isolates were obtained from the Masan National TB Hospital.

### Genomic DNA extraction

Genomic DNA of clinical isolates was extracted using the chloroform-isoamyl alcohol method, as previously described (10).

### PCR primers and oligonucleotide probes for microplate hybridization assay

The design of primers and probes used in this assay was based on the sequences of *M. tuberculosis* H37Rv obtained from GeneBank (http://www.ncbi.nlm.nih.gov/entrez/viewer.fcgi?db=Nucleotide&dopt=GenBank&val=41352722). The target sequences involved in *katG* and *inhA* mutation refer to Ramaswamy *et al* (5). Two sets of primers for PCR amplification and for sequencing were designed to amplify the *katG* 315 gene and 15 UPS. Both the reverse primers were biotin-labelled at 5' end. The primers were *katG* 315 F (AGAGCT CGTATGGCACCGGA) and *katG* 315R* (CCAGCAGGGCTCT TCGTCAG) for *katG* region, and *inhA*-1F (ACGCTCGTGGCAT ACCGATT) and *inhA*-1R* (TCACATTCGACGCC AAACAG) for 15UPS region. Four probes were used for microplate hybridization assay. Two probes, *katG* 315 WT (TCACCA**G**CGGCAT CGAGG) and 15 UPS WT (CCGCGGCGAGA**C**GATAGG), were designed to detect the wild type genotypes as each own, respectively whilst two further probes, *katG* 315 MT (GATCAC CA**C**CGGCATCGAGG) and 15 UPS MT (CCGCGGCGAGA**T**G ATAGG), were designed to detect the most frequently seen genotype at each locus, *katG* 315 $^{AGC \rightarrow ACC}$ and 15 UPS $^{C \rightarrow T}$, respectively. All the oligonucleotide probes were labeled at the 5' end with digoxigenin. The nucleotide sequences of the primers and the probes were analyzed with the BLAST search (http://www.ncbi.nlm.nih.gov) to confirm the uniqueness of

each primer and probe.

Biotin-labeled and unlabeled PCR primers and digoxigenin linked oligonucleotide probes for microplate hybridization assay were synthesized by the Bioneer (Daejeon, Korea) and Sigma-Proligo (The Woodlands, TX, USA), respectively.

### PCR amplification

Biotinylated target PCR products were generated in Accu-Power PCR PreMix (Bioneer, Daejeon, Korea) containing Taq DNA polymerase 2.5 U; dNTP (dATP, dCTP, dGTP, dTTP) 250 uM; Tris-HCl (pH 9.0) 10 mM; KCl 40 mM; $MgCl_2$ 1.5 mM; stabilizer and tracking dye. Ten picomol of each primer and 2 µl of the extracted DNA (1-10 ng/µl) were added to PCR PreMix tube. PCR was performed with initial denaturation step of 95°C for 5 min before 35 cycles of 95°C for 30 s, 58°C for 30 s and 72°C for 30 s and then a final step of 72°C for 7 min for the last cycle (model GeneAmp® PCR system 2700, Perkin-Elmer Biosystems).

### DNA sequence analysis

This was conducted using Genotech (Daejeon, Korea) with the primers used for PCR amplification. The Chromas software was used to analyze the sequencing data obtained from Genotech (http://www.technelysium.com.au/chromas14x.html).

### Microplate hybridization assay

Microplate hybridization assay was performed using the following protocols. Briefly, in order to prepare the sample, amplified PCR product was diluted 4 fold with purified water. Each diluted double strand-PCR product was added to the Streptavidin coated microplate (Nunc, nunc immobilizer™ streptavidin, Denmark) wells. To hybridize, after denaturation of the double strand-PCR product, each diluted probe was added to the wells and incubated at 60°C for 1 hour. The microplate was then washed three times with washing buffer at 60°C for 10 min with 50 rpm shaking, and then incubated with anti-Digoxigenin-peroxidase (Roche, no. 11 207 733 910). After the plate wells were washed with washing buffer, substrate solution containing equal volume of TMB (3,3',5,5'-tetrametyl benzidine, sigma, no. T0440) and 0.1% $H_2O_2$ was added and color was allowed to develop for 10 min. Finally, the reaction was stopped with 1 N HCl. The absorbance value of the reaction mixture at 450 nm was determined using a microplate reader (Molecular Devices, U.S.A).

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